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Features of the *mcr-1* cassette respect to colistin resistance

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The recent description of the plasmid-mediated colistin resistance gene, *mcr-1*, in strains isolated from food animals, food and humans in China was a signal for an avalanche of retrospective and prospective studies investigating the occurrence of this specific gene (1). The *mcr-1* gene has been identified almost all over the world now, and the earliest evidence for its presence dates back to the 1980's (2). The *mcr-1* gene has so far been associated with non-related types of plasmid replicons such as IncI2, IncHI2, IncP, IncFIB and IncX4 (1,3-5) and was found only rarely to be chromosomally encoded (6). This gene is part of a 2,600-bp long fragment designed as the *mcr-1* cassette that encompasses the likely promoter sequences for *mcr-1* expression (7). The *mcr-1* gene is most often located at the right-hand extremity of the insertion element IS*Apl1*, together with a 723-bp long open reading frame (named *orf723*) encoding an hypothetical protein. According to blast analysis, it is a putative phosphoesterase, and shares 45% and 44% identities with those of *Corynebacterium durum* (Accession-Nr: WP_060996190.1) and *Psychrobacter arcticus* (Accession-Nr: WP_011280438.1), respectively. However, the putative contribution of this orf for expression of the *mcr-1* gene and subsequently to colistin resistance remains unknown.

Our goal was to evaluate the role of *orf723* with respect to the colistin resistance. Therefore, three *Escherichia coli* recombinant strains were constructed, with the same plasmid harbouring either the *mcr-1* gene alone, *orf723* alone, and the entire *mcr-1* cassette, respectively. The primers used to amplify the *mcr-1* gene, the 723-bp orf and the whole *mcr-1* cassette are listed in Table 1, and the *mcr-1*-positive *E. coli* OW3E1 (GenBank Accession number: KX129783) was used as template. Amplicons were double-digested with restriction enzymes BamHI and EcoRI and cloned into the low-

copy vector pCCR9 (Taxonomy ID: 125570) digested with the respective enzymes, to create vectors pCCR9::mcr-1, pCCR9::orf723 and pCCR9::mcr-1::orf723. The constructs were transformed by electrotransformation into *E. coli* DH5α, giving rise to recombinant strains DH5α::pCCR9::mcr-1, DH5α::pCCR9::orf723, and DH5α::pCCR9::mcr-1::orf723, respectively. Minimal inhibitory concentrations (MIC) of colistin were determined using broth dilution tests as recommended by EUCAST.

MICs are summarized in Table 2. MIC values of recombinant strains expressing MCR-1 with and without the *orf723* were increased and identical. These results further confirm that expression of the *mcr-1* gene confers reduced susceptibility to colistin. However they show that *orf723* encoding an hypothetical protein and which has likely been co-mobilized with the *mcr-1* gene from its original genetic context does not impact colistin susceptibility.

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Transparency declarations

None to declare

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Table 1: Primers used for cloning of *mcr-1*, the hypothetical protein and the whole *mcr-1* cassette

Primer name	Sequence (5'-3')[a]	Tm (°C)[b]	Location
mcr_BamHI_up	TTTTTTGGATCCGCCGCAATTATCCCACCG	53	22 bp upstream of *mcr-1* start codon[c]
mcr_EcoRI_dn	TTTTTTGAATTCCCACCGCCCATAATACGAATGG	56	36 bp downstream of *mcr-1* stop codon
orf723_BamHI_up2	TTTTTTGGATCCGCACACTCCATTCGTATTATGGGC	57	18 bp upstream of 723 bp orf start codon
orf723_EcoRI_dn	TTTTTTGAATTCCCGTTCCTATTGGTAGTTTCCAGG	56	81 bp downstream of 723 bp orf stop codon

[a] The restriction sites are underlined.
[b] T_m, melting point.
[c] downstream of putative promotor region.

Table 2: Minimal inhibitory concentrations (MIC's) of colistin for each *E. coli* DH5α conjugants as well as the negative controls *E. coli* DH5α::pCCR9 and *E. coli* DH5α using broth dilution tests as recommended by EUCAST

Conjugant / Strain	**MIC colistin [mg/L]**
DH5α::pCCR9::mcr-1	4
DH5α::pCCR9::orf723	1
DH5α::pCCR9::mcr-1::orf723	4
DH5α::pCCR9	0.5
DH5α	1